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14. ABSTRACT

The basal-like breast cancer (BLBC) subtype accounts for a high percentage of overall breast cancer mortality. The current therapeutic options for BLBC are ineffective; hence, elucidating signaling pathways that drive BLBC growth may identify novel targets for the development of effective therapies. Rho GTPases have previously been implicated in promoting tumor cell proliferation and metastasis. These proteins are inactivated by GTPase-activating proteins (GAPs), which have generally been presumed to act as tumor suppressors. Surprisingly, microarray analysis for the expression of Rho GTPase regulators revealed significant upregulation of several RhoGAP genes in BLBCs. The aim of our research is to characterize the role of two of these RhoGAPs, ArhGAP11A and RacGAP1, in BLBC development. In BLBC cell lines, shRNA-mediated knockdown of ArhGAP11A resulted in significant proliferation defects that were caused via $p27^{Kip1}$-mediated induction of cell cycle arrest. ArhGAP11A was also found to control cell spreading and migration. RacGAP1 depletion significantly retarded BLBC growth but, in contrast to ArhGAP11A, this was due to the combined effects of cytokinesis failure, $p21^{WAF1/Cip1}$-mediated pRb inhibition, and the onset of senescence. In summary, we have established that ArhGAP11A and RacGAP1 are both critical to in vitro BLBC growth, consistent with an oncogenic role for these GAPs.

15. SUBJECT TERMS

ArhGAP11A, RacGAP1, basal-like breast cancer, RhoGAPs, Rho GTPases, RhoA, Rac1, Cdc42, proliferation, migration

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1. INTRODUCTION:

The basal-like subtype of human breast cancer accounts for a disproportionately high percentage of overall breast cancer recurrence and death, and the current therapeutic options for this cancer are ineffective. Hence, elucidating the signaling pathways that are responsible for driving the growth of basal-like tumors may identify novel targets for the development of effective therapies. Rho family small GTPases have previously been implicated in promoting tumor cell proliferation, invasion, and metastatic growth in a variety of cancers. These proteins are activated by guanine nucleotide exchange factors (GEFs) and, in the context of cancer, overexpressed RhoGEFs can function as oncogenes which cause hyper-elevated Rho GTPase activity. In contrast, GTPase-activating proteins (GAPs), which return Rho GTPases to an inactive, GDP-bound state, have generally been presumed to act as tumor suppressors. Surprisingly, microarray analysis of the expression of Rho GTPases, GEFs, and GAPs across a panel of human breast tumors revealed that a number of RhoGAP genes were significantly upregulated in basal-like breast cancers (BLBCs). These preliminary results suggested that RhoGAPs may play an unexpected role in promoting tumor growth. The aim of our research is therefore to validate and characterize the role of two of these RhoGAPs, ArhGAP11A (also known as MP-GAP) and RacGAP1 (also known as MgcRacGAP and CYK4), in BLBC development.

2. KEYWORDS: ArhGAP11A, RacGAP1, basal-like breast cancer, RhoGAPs, Rho GTPases, RhoA, Rac1, Cdc42, proliferation, migration

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project, as described in the approved Statement of Work, were as follows:

Major Task 1. Validate the role of RhoGAPs in BLBC oncogenesis

a) *In vitro* tumor growth and invasion assays.
b) Western blots for ArhGAP11A and RacGAP1 protein expression in human tumor samples, human cell lines, and mouse models.
c) *In vivo* tumorigenesis and metastasis assays.

Milestones: Identify whether ArhGAP11A and RacGAP1 can promote tumor growth and/or invasion both *in vitro* and *in vivo* (by month 12). **Approximately 70% completed by month 12.**

Major Task 2. Determine the functions of ArhGAP11A and RacGAP1

a) Proliferation, cytokinesis, and apoptosis assays.
b) Transformation assays.
c) Migration analyses.

Milestones: Identify the specific functions of ArhGAP11A and RacGAP1 in BLBC tumorigenesis or metastasis (by month 24). **Approximately 80% completed by month 12.**

Major Task 3. Determine if ArhGAP11A and RacGAP1 promote tumorigenesis in a GAP activity-dependent manner

a) Express and purify isolated GAP domains and catalytically-inactive mutants of ArhGAP11A and RacGAP1.

b) GAP activity assays.
c) Rho GTPase pulldown assays.
d) Rescue experiments with wild type or catalytically-inactive GAPs.

Milestones: Identify the GTPase specificity of ArhGAP11A and determine if disrupted GTPase activity is responsible for the tumorigenic phenotypes of ArhGAP11A- or RacGAP1-depleted cells (by month 36). **Approximately 20% completed by month 12.**

What was accomplished under these goals?

- **Major Activities and Specific Objectives**

The major research activities undertaken in the last 12 months have been to validate the role of ArhGAP11A and RacGAP1 in BLBC oncogenesis and to determine the function of these RhoGAPs in BLBC cell lines. SUM149 and HCC1937 were identified to be appropriate human BLBC cell lines to use for this study, as both exhibited high protein expression levels of the two RhoGAPs in question. Protocols to efficiently knockdown ArhGAP11A or RacGAP1 from these cell lines, using lentivirally-delivered shRNA constructs, were then established. Successful depletion of these proteins has allowed us to pursue the major objective of identifying whether loss of expression of either ArhGAP11A or RacGAP1 in BLBC cell lines leads to changes in cellular function that are consistent with a role for these GAPs in promoting BLBC tumorigenesis. More specifically, we have assessed the ability of SUM149 and HCC1937 cell lines to proliferate in vitro in the absence of these GAPs (using 2D clonogenic and/or MTT proliferation assays) and, having identified a growth defect, have gone on to characterize the mechanisms through which growth is inhibited in either case, by performing apoptosis, cytokinesis, cell cycle, and senescence assays. In addition, fluorescent microscopy and time-lapse imaging have been used to identify defects in the ability of ArhGAP11A- and RacGAP1-depleted cells to spread and migrate.

- **Key Outcomes**

Preliminary data (included in the original project proposal) indicated that ArhGAP11A and RacGAP1 mRNA levels were significantly upregulated in human basal-like breast tumors as compared to normal-like breast tissue and to the luminal subtype with good prognosis (LumA) (Fig. 1A-B). Both RhoGAPs were also upregulated in basal (C3(I)-Tag) but not luminal (MMTV-Neu) genetically-engineered mouse models (Fig. 1B). At the protein level, RacGAP1 was found to be overexpressed in tumors from basal (C3(I)-Tag) mice relative to both neighboring normal tissues and to tumors from luminal (MMTV-Neu) mice (Fig. 1E). Similarly, RacGAP1 protein levels were higher in BLBC cell lines, relative to normal-like or luminal breast cancer cell lines (Fig. 1D). Subsequent experiments (in the last 12 months) have built upon these preliminary results to verify that ArhGAP11A protein is also typically expressed at higher levels in human BLBC cell lines than in other subtypes, as determined by western blot analyses (Fig. 1C). Since SUM149 and HCC1937 cells expressed relatively high protein levels of both ArhGAP11A and RacGAP1, we elected to use these two BLBC cell lines in our functional studies to validate a role for these GAPs in BLBC tumorigenesis.

To date, we have used a stable-knockdown approach to determine the biological function of these GAPs in BLBC. From an initial panel of five shRNAs per gene, we identified the two constructs that reduced the expression of each GAP to the greatest extent following lentivirally-mediated delivery into SUM149 or HCC1937 cells and puromycin selection. Western blot analyses indicated that for ArhGAP11A, the sh3 and sh5 vectors gave the strongest knockdown (typically reducing ArhGAP11A

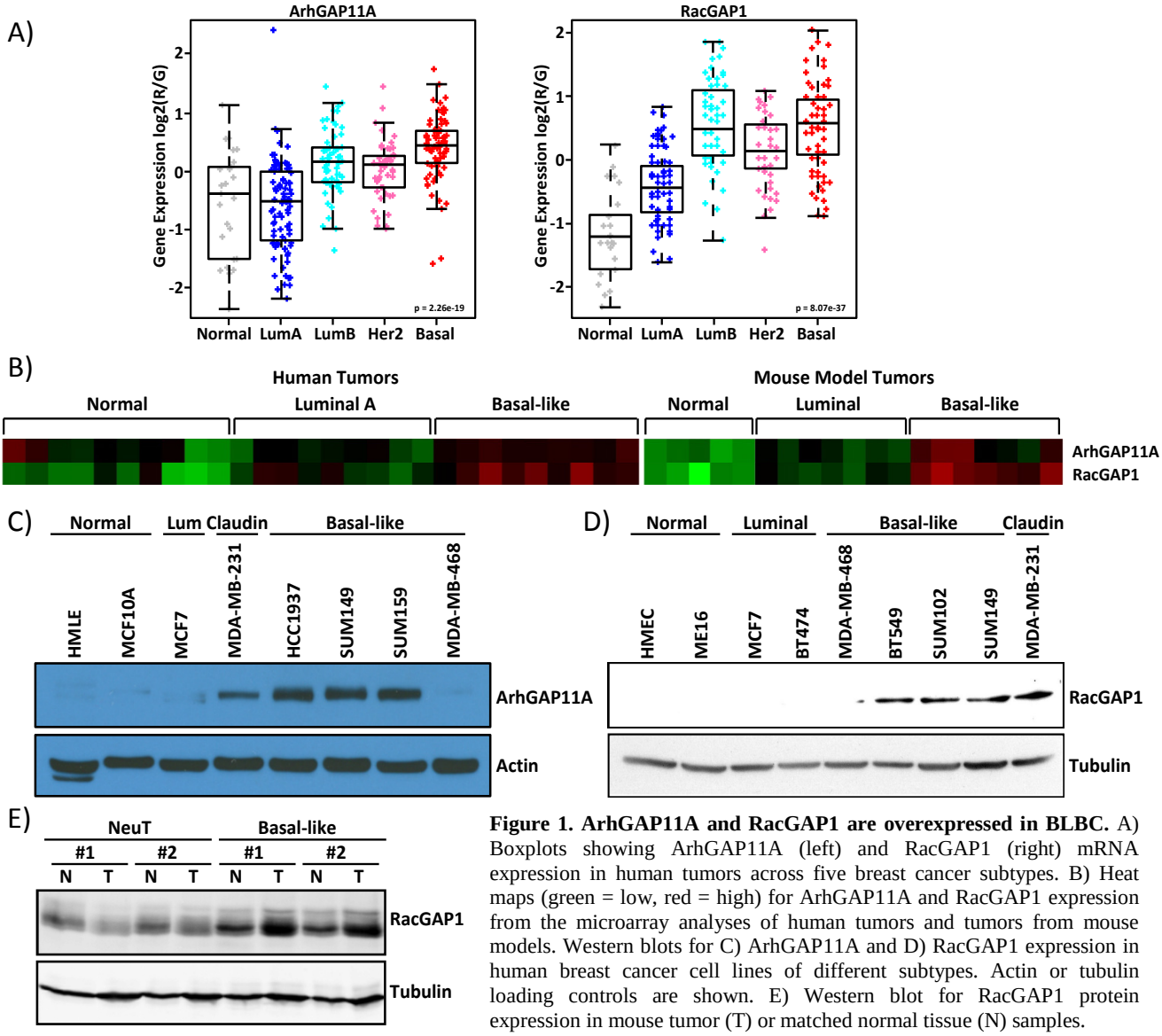

Figure 1. ArhGAP11A and RacGAP1 are overexpressed in BLBC. A) Boxplots showing ArhGAP11A (left) and RacGAP1 (right) mRNA expression in human tumors across five breast cancer subtypes. B) Heat maps (green = low, red = high) for ArhGAP11A and RacGAP1 expression from the microarray analyses of human tumors and tumors from mouse models. Western blots for C) ArhGAP11A and D) RacGAP1 expression in human breast cancer cell lines of different subtypes. Actin or tubulin loading controls are shown. E) Western blot for RacGAP1 protein expression in mouse tumor (T) or matched normal tissue (N) samples.

protein expression in both cell lines by ~90% and ~60%, respectively, relative to a non-silencing (NS) control) (Fig. 2A). For RacGAP1, knockdown was most efficient with constructs sh1 and sh2 (both ~80%) (Fig. 2B). Having established knockdown of both ArhGAP11A and RacGAP1 in two BLBC cell lines, we then assessed how knockdown of these GAPs affected tumor cell growth in vitro.

Cells were first subjected to 2D clonogenic assays. Strikingly, SUM149 or HCC1937 cells lacking either ArhGAP11A or RacGAP1 formed significantly fewer colonies relative to the NS control over the same time frame (Fig. 3A-B), suggesting that both GAPs are required for the efficient growth of these BLBC cell lines. To further characterize the growth defect, the proliferation of ArhGAP11A- and RacGAP1-depleted SUM149 cells was monitored using MTT viability assays. Again, significant defects in growth were observed in each case. Whereas parental (SUM149) and NS cells exhibited comparable growth dynamics, cells treated with ArhGAP11A sh3 or RacGAP1 sh2 completely failed to proliferate. ArhGAP11A sh5 and RacGAP1 sh1 cells also underwent growth arrest after relatively slow growth for 2-3 days (Fig. 3C). The finding that BLBC cells that lack ArhGAP11A or RacGAP1 fail to proliferate in vitro suggests that the usual function of these GAPs in BLBC is to promote growth. These results therefore support the hypothesis that these RhoGAPs are indeed playing an oncogenic role in these cells.

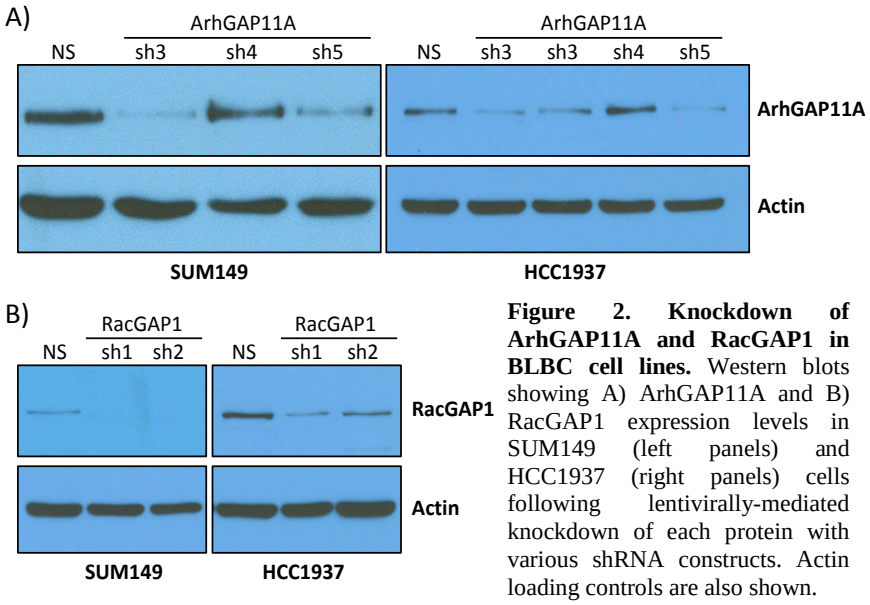

Figure 2. Knockdown of ArhGAP11A and RacGAP1 in BLBC cell lines. Western blots showing A) ArhGAP11A and B) RacGAP1 expression levels in SUM149 (left panels) and HCC1937 (right panels) cells following lentivirally-mediated knockdown of each protein with various shRNA constructs. Actin loading controls are also shown.

The next step in identifying the function(s) of ArhGAP11A and RacGAP1 was to delineate the mechanism(s) through which each GAP supported BLBC proliferation. This has been accomplished by performing apoptosis, cytokinesis, cell cycle, and senescence assays. Apoptosis of GAP-depleted SUM149 cells was assayed by western blot analyses for two apoptotic markers, cleaved PARP and caspase 3. However, no substantial differences were observed in the levels of these cleaved proteins in cells lacking either GAP, relative to control cells (Fig. 4A-B). This finding, coupled to observations from fluorescent microscopy and cell cycle flow cytometry analyses, suggests that apoptosis is not responsible for causing the proliferation defect that is characteristic of ArhGAP11A- and RacGAP1-depleted cells.

RacGAP1 has a well-documented role in regulating cytokinesis[1-5], the final stage of cell division, and ArhGAP11A has also recently been implicated in the control of this process in HeLa cells[6]. To investigate the possibility that defects in cytokinesis may be responsible for the inability of either ArhGAP11A- or RacGAP1-depleted cells to proliferate, we examined the ability of GAP-deficient SUM149 cells to efficiently divide. Defects in cytokinesis result in the formation of bi- or multinucleated cells, which were identified and quantitated using fluorescent microscopy. Consistent with a role for RacGAP1 in regulating cytokinesis, we observed that ~30-40% of SUM149 cells became bi- or multinucleated upon RacGAP1 knockdown (Fig. 5). Hence, cytokinesis failure is likely to make a relatively large contribution to the inability of RacGAP1-depleted cells to proliferate. In contrast, knockdown of ArhGAP11A only resulted in ~11% of cells failing cytokinesis and becoming bi/multinucleated (Fig. 5). Although this slight defect may partially contribute to growth impairment, we suspect that it is insufficient to account for the substantial growth defects observed upon knockdown of ArhGAP11A (Fig. 3).

Having ruled out apoptosis and cytokinesis failure as factors that make a major contribution to the growth defect of ArhGAP11A-deficient cells, we next performed flow cytometry analysis of propidium iodide-stained cells to identify whether GAP-deficient SUM149 cells become arrested at a specific phase of the cell cycle. This analysis revealed that, relative to NS cells, ArhGAP11A-deficient cells accumulated in the G1 phase of the cell cycle, as shown by the decreased proportion of cells that entered the S or G2/M phases (Fig. 6A-B). This defect was stronger with ArhGAP11A sh3 than sh5 (Fig. 6B), which may reflect the relative levels of knockdown with each construct (Fig. 2). The finding that ArhGAP11A is required for efficient cell cycle progression is novel and indicative of a pro-tumorigenic role for this GAP in BLBC.

We next performed western blot analyses for proteins that are involved in the G1 to S phase cell cycle transition, with the aim of elucidating the molecular mechanisms responsible for causing G1 arrest in ArhGAP11A-depleted cells. The key finding of this analysis was that the Rb tumor suppressor, which allows G1/S transition when inactivated by hyperphosphorylation, had dramatically reduced

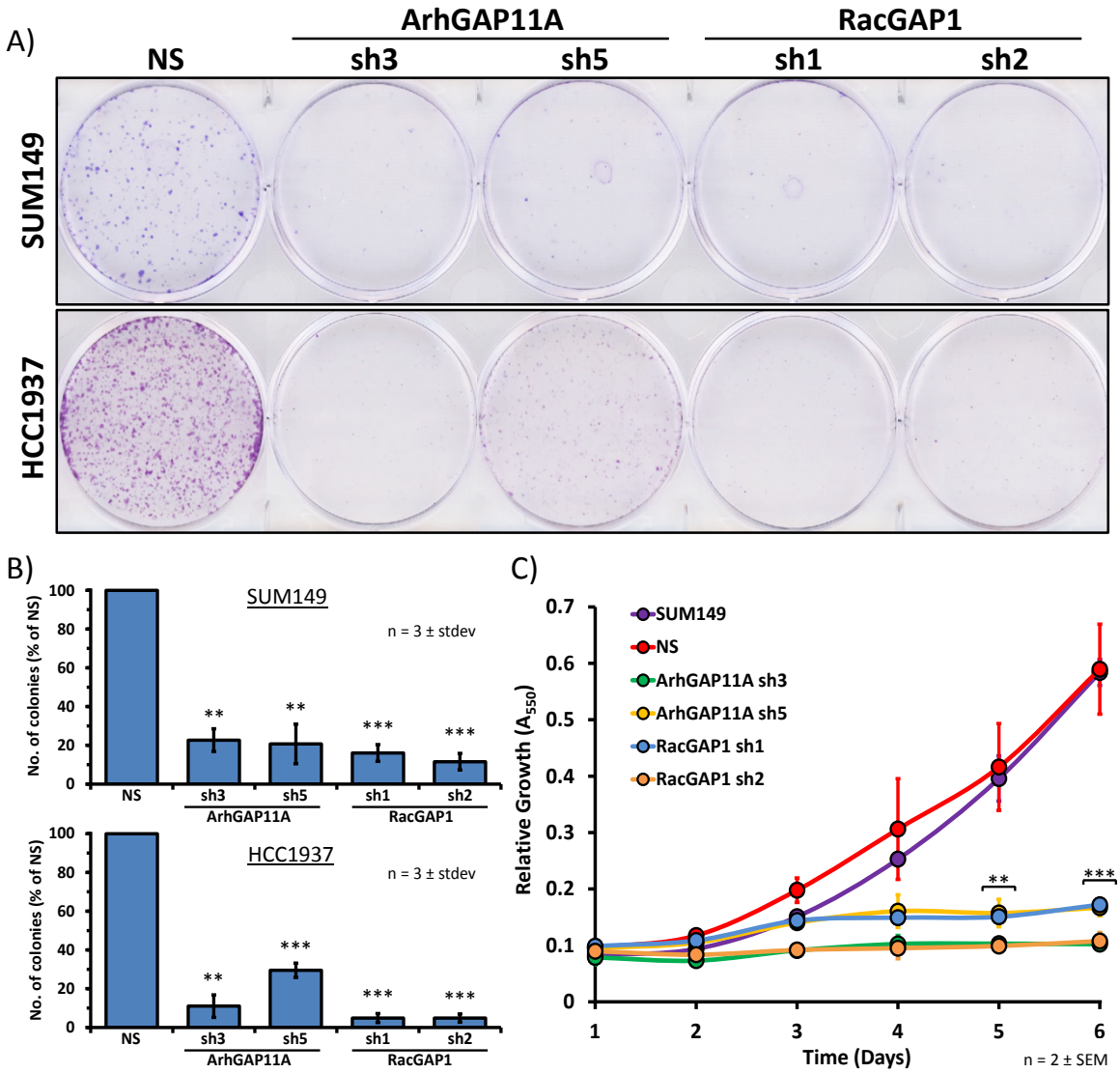

Figure 3. ArhGAP11A and RacGAP1 are both required for BLBC proliferation in vitro. A) Representative images of crystal violet-stained 2D clonogenic assays with SUM149 (upper) and HCC1937 (lower) cells after 7 or 10 days growth, respectively. B) Quantification of 2D colony formation, normalized to NS control. C) MTT assay showing SUM149 cell proliferation over time with or without knockdown of ArhGAP11A or RacGAP1.

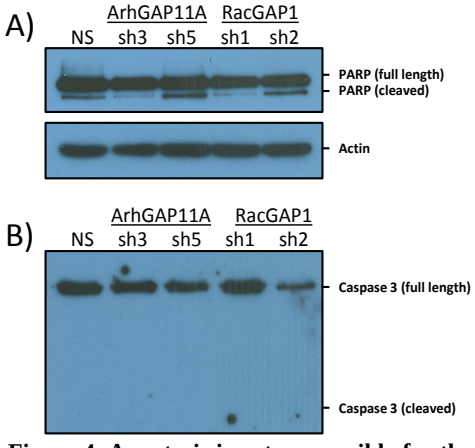

Figure 4. Apoptosis is not responsible for the growth defect of ArhGAP11A- or RacGAP1-depleted cells. Western blots for A) PARP or B) caspase 3 cleavage.

phosphorylation upon knockdown of ArhGAP11A (Fig. 6C). Phosphorylation of Rb is controlled by cyclin-dependent kinases (CDKs) in complex with cyclins, but can be inhibited by CDK inhibitors such as $p16^{INK4A}$, $p21^{WAF1/Cip1}$, or $p27^{Kip1}$. Notably, high expression of $p27^{Kip1}$ was detected in lysates of SUM149 cells depleted of ArhGAP11A (Fig. 6C). As neither $p21^{WAF1/Cip1}$ (Fig. 6C) nor $p16^{INK4A}$ (which is not expressed in SUM149 cells) were upregulated in ArhGAP11A-depleted cells, our results suggest that $p27^{Kip1}$ was the CDK inhibitor responsible for the hypophosphorylation of Rb and the associated arrest in G1 that occurred upon ArhGAP11A knockdown. This $p27^{Kip1}$-mediated growth arrest is likely to be the major mechanism through which ArhGAP11A-depleted cells fail to proliferate.

In contrast to ArhGAP11A knockdowns, RacGAP1-

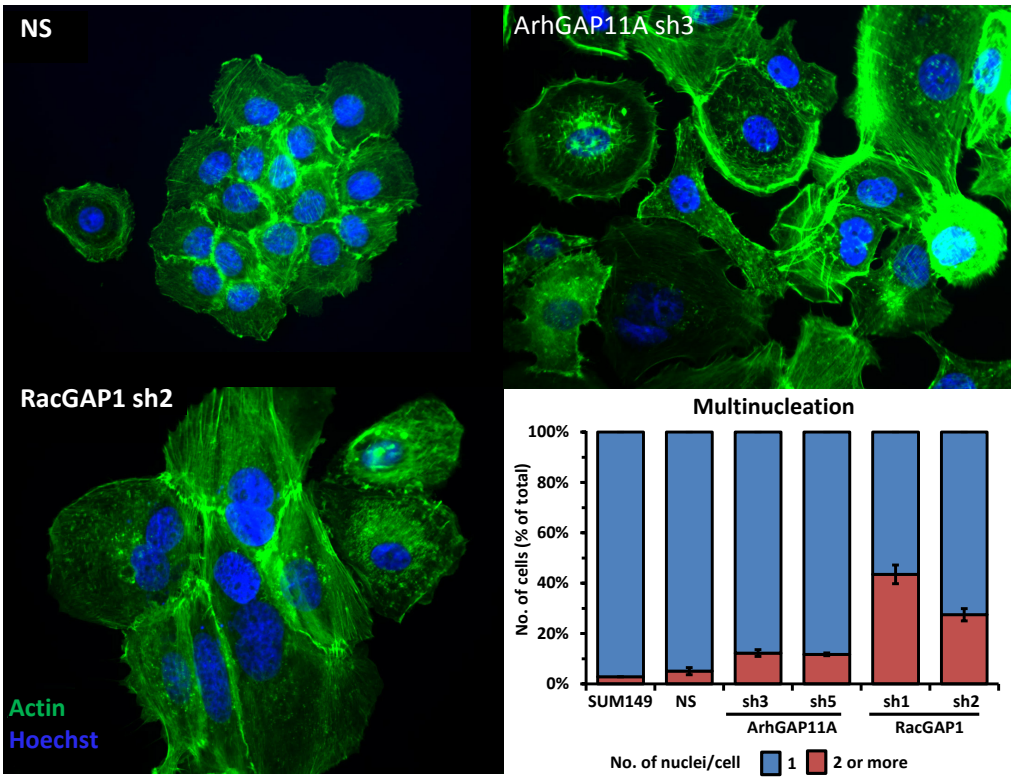

Figure 5. RacGAP1 knockdown results in cytokinesis failure. Representative fluorescent images showing actin (green) and Hoechst (blue) staining of NS (upper left), ArhGAP11A sh3 (upper right), and RacGAP1 sh2 (lower left) SUM149 cells. Images are on the same scale. The percentage of bi/multinucleated cells in each population is quantified (lower right).

deficient cells did not exhibit a markedly altered cell cycle progression pattern relative to the NS control (Fig. 6B). Despite this, RacGAP1-depleted cells also had very low levels of Rb phosphorylation (Fig. 6C). In these cells, the CDK inhibitor $p21^{WAF1/Cip1}$ was upregulated (Fig. 6C), emphasizing that different pathways were activated in response to the loss of RacGAP1 as compared to ArhGAP11A. The $p21^{WAF1/Cip1}$-mediated inhibition of pRb may contribute to the growth defect of RacGAP1-depleted cells, although it is currently unclear whether this occurs as a result of, or in addition to, the defect in cytokinesis. The presence of multinucleated cells complicates the analysis of the flow cytometry data and may explain why no cell cycle defect was detected in samples lacking RacGAP1.

Interestingly, by staining for senescence-associated β-galactosidase expression, SUM149 cells lacking RacGAP1 were found to have enhanced levels of senescence (~9-11% of the total population) relative to control cells (~2% senescent) (Fig. 6D). ArhGAP11A knockdown did not greatly affect senescence levels (Fig. 6D), suggesting that RacGAP1-associated phenotypes (multinucleated cells, $p21^{WAF1/Cip1}$ upregulation) may promote senescence, whereas the $p27^{Kip1}$-mediated cell cycle arrest of ArhGAP11A-depleted cells does not.

In addition to studying the effects of ArhGAP11A and RacGAP1 on BLBC proliferation, we have examined the role of these GAPs in regulating cell spreading and migration, two processes that are important for cancer development and that are known to be reliant on Rho GTPase-dependent cytoskeletal dynamics. The depletion of either ArhGAP11A or RacGAP1 from SUM149 cells caused mononucleated cells to spread on fibronectin with an approximately 30-50% larger area than control cells, as assessed using fluorescent microscopy (Fig. 7A-B). Enhanced spreading was also observed on uncoated glass coverslips (Figs. 5, 7C). As multinucleated cells typically exhibit greatly increased spread areas, these cells were excluded from the spreading analysis. These results suggest that both ArhGAP11A and RacGAP1 are involved of the regulation of cell spreading, which would indicate that one of their functions may be to regulate the cytoskeleton via Rho GTPase signaling. However, the

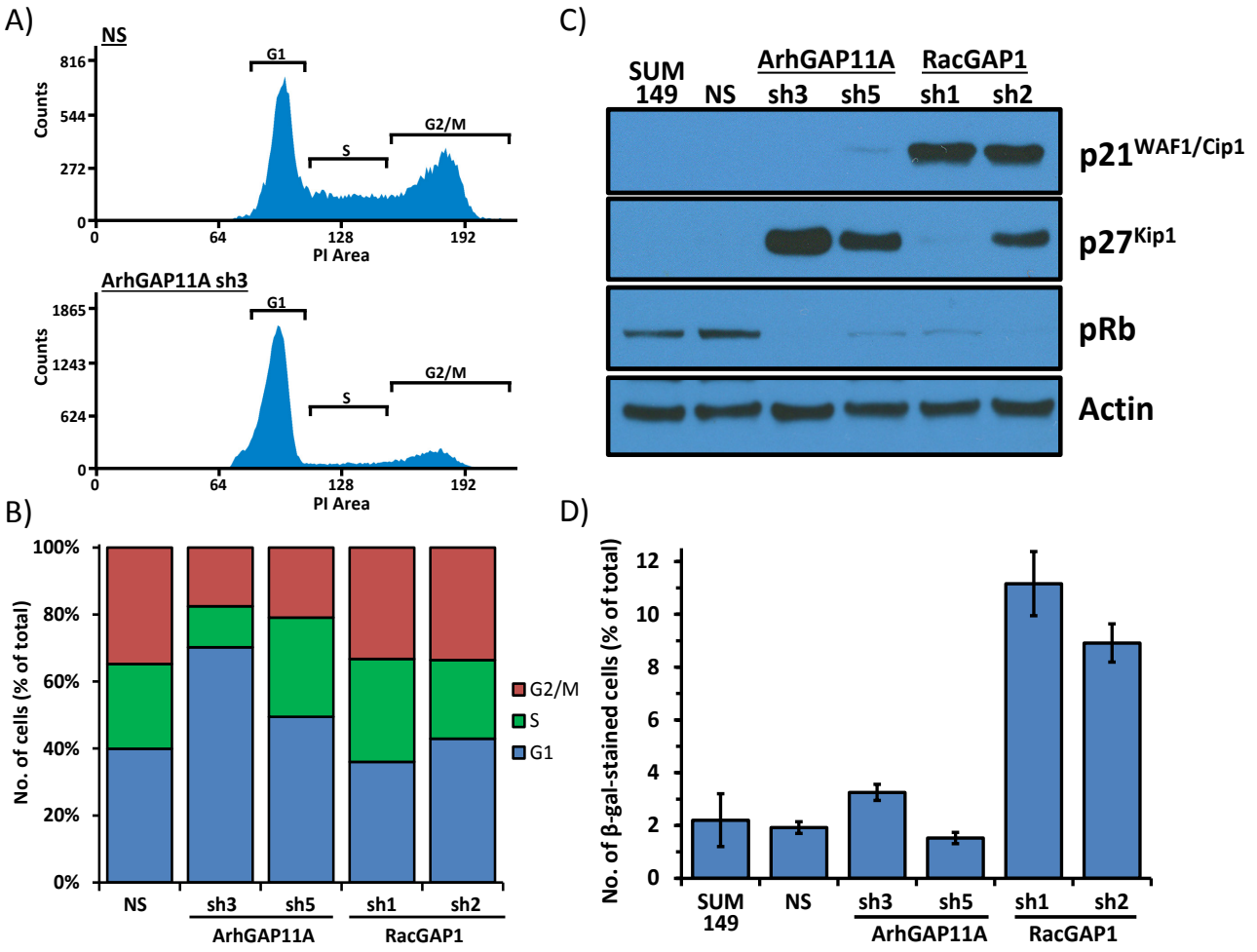

Figure 6. ArhGAP11A-depleted cells undergo p27^{Kip1}-mediated cell cycle arrest. A) Representative histograms of propidium iodide (PI)-stained NS (upper) and ArhGAP11A (lower) cells as identified using flow cytometry. The relative boundaries of G1, S, and G2/M phases are indicated. B) Quantification of the percentage of cells in each phase of the cell cycle upon GAP knockdown. C) Western blots for p21$^{WAF1/Cip1}$, p27^{Kip1}, pRb, and actin levels in control and GAP-depleted SUM149 cells. D) Quantification of the percentage of senescent cells in each population, as identified by staining for senescence-associated β-galactosidase (β-gal).

possibility that the spread morphologies of these cells may be secondarily linked to the onset of senescence (particularly in the case of RacGAP1-depleted cells) cannot be excluded.

The ability of tumor cells to migrate is critical to their ability of metastasize in vivo. We have studied the migration of GAP-depleted SUM149 cells in vitro by performing time-lapse microscopy experiments on randomly migrating cell populations, in collaboration with Dr. James Bear's lab at UNC. By tracking the movement of individual cells over a 24 h period, we have identified a migration defect in cells that lack ArhGAP11A. The average velocity of ArhGAP11A sh3 cells is significantly (~40%) lower than that of NS cells (Fig. 7D). Again, the phenotype of the ArhGAP11A sh5 cells is not as strong, most likely due to the differing extent of knockdown. Surprisingly, RacGAP1-depleted cells had no defects in migration velocity (Fig. 7D), despite their increased spread area. These results indicate that ArhGAP11A, but not RacGAP1, promotes the migration of BLBC cells in vitro, which may influence the ability of BLBC tumors to metastasize.

In summary, research performed over the past 12 months has produced several key outcomes with respect to validating and characterizing the functions of ArhGAP11A and RacGAP1 in BLBC. Our experimental results reveal that ArhGAP11A is overexpressed in BLBC at both the mRNA and at the protein level, and that loss of this GAP abrogates the ability of BLBC cell lines to proliferate in vitro. This appears to be caused via a p27^{Kip1}-mediated induction of cell cycle arrest. In addition to promoting

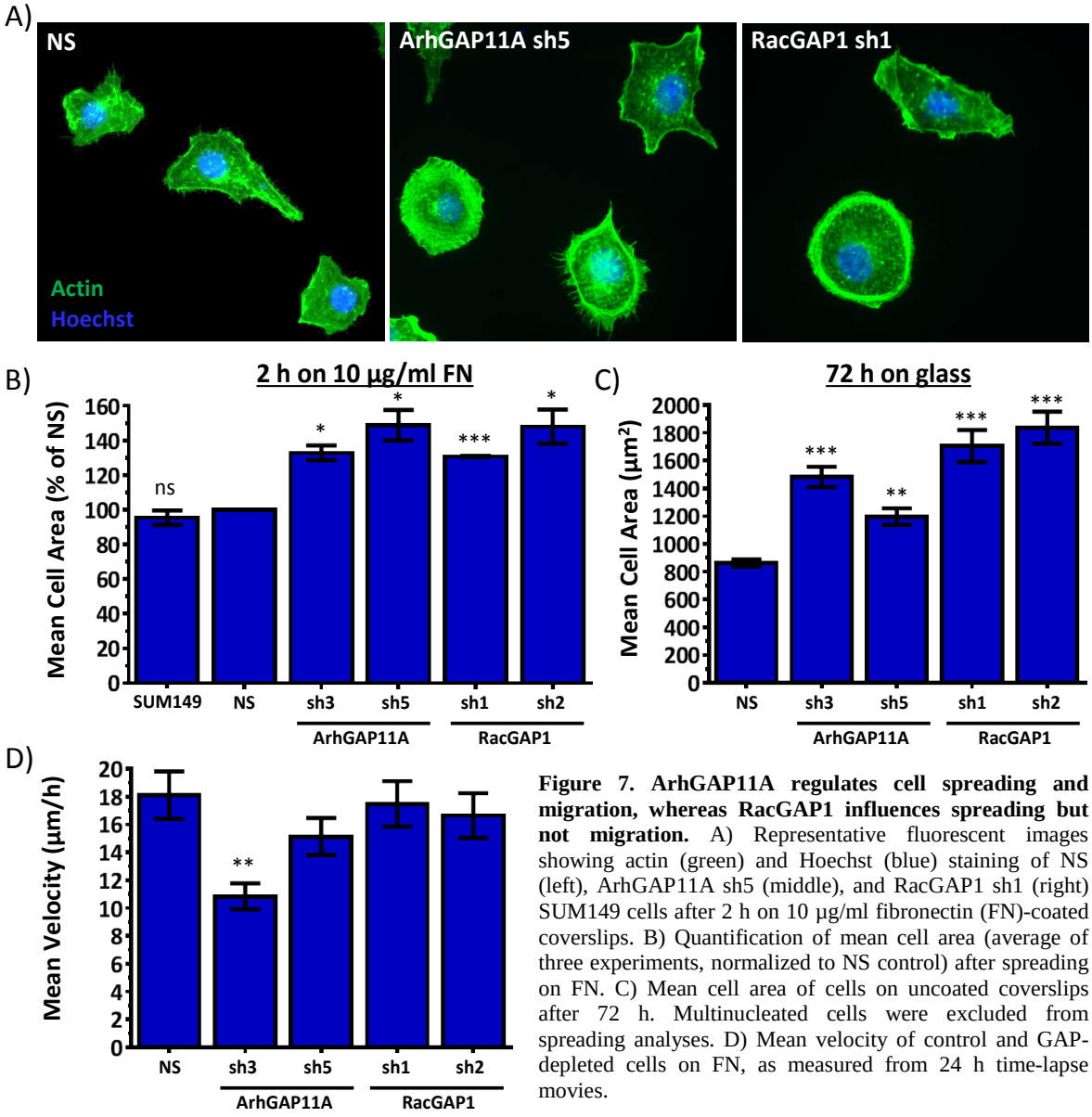

Figure 7. ArhGAP11A regulates cell spreading and migration, whereas RacGAP1 influences spreading but not migration. A) Representative fluorescent images showing actin (green) and Hoechst (blue) staining of NS (left), ArhGAP11A sh5 (middle), and RacGAP1 sh1 (right) SUM149 cells after 2 h on 10 µg/ml fibronectin (FN)-coated coverslips. B) Quantification of mean cell area (average of three experiments, normalized to NS control) after spreading on FN. C) Mean cell area of cells on uncoated coverslips after 72 h. Multinucleated cells were excluded from spreading analyses. D) Mean velocity of control and GAP-depleted cells on FN, as measured from 24 h time-lapse movies.

cancer cell growth, ArhGAP11A has been identified to control cell spreading and migration. These findings indicate that ArhGAP11A may indeed be acting as an oncogene in BLBC.

Like ArhGAP11A, RacGAP1 is also expressed at high levels in BLBC and cells cease to proliferate upon its loss. In contrast to ArhGAP11A, the proliferation defect of cells lacking RacGAP1 is most likely due to the combined effect of a reduced ability to complete cytokinesis, the $p21^{WAF1/Cip1}$-mediated inhibition of pRb, and the onset of senescence. RacGAP1 does appear to regulate spreading but not the migration of BLBC cells. These phenotypes do support a role for RacGAP1 in promoting BLBC, but it is currently unclear to what extent these can be explained by defects in cytokinesis alone.

An important future aim of this project is to identify the precise mechanisms through which each GAP promotes the phenotypes identified to date. A critical future direction is to identify whether each phenotype can be accounted for by changes in the activity levels of Rho GTPases. In vitro GAP assays performed by Dr. Kent Rossman in the Der lab, in addition to data published by other labs, indicate that RacGAP1 is specific for the Rho-family GTPases Rac1 and Cdc42[3], whereas ArhGAP11A acts predominantly on RhoA[6-8], but also on Cdc42 (Fig. 8). Pulldown analyses to monitor changes in the GTP-bound, activated levels of RhoA, Rac1, and Cdc42 in ArhGAP11A- and RacGAP1-depleted cells are currently underway.

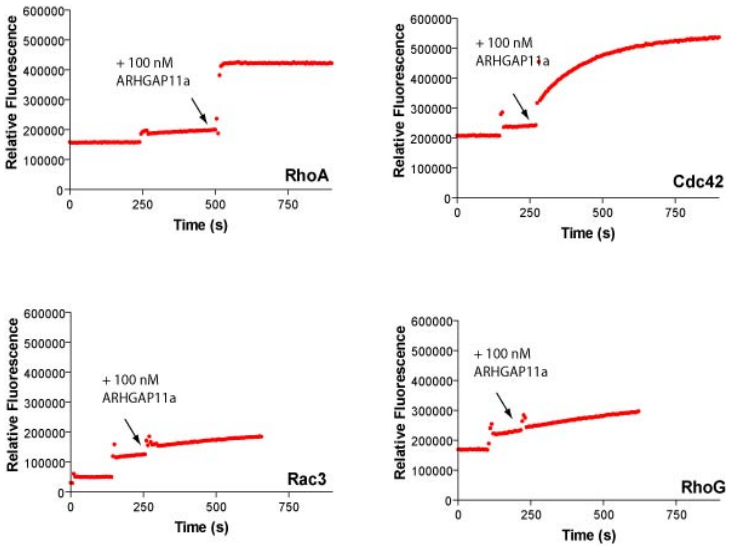

Figure 8. ArhGAP11A is a RhoA- and Cdc42-specific GAP. In vitro GAP assays, demonstrating the ability of the purified GAP domain of ArhGAP11A to hydrolyze GTP bound to RhoA and Cdc42, but not Rac3 or RhoG. The intrinsic rate of GTP hydrolysis by each GTPase is shown prior to the addition of 100 nM ArhGAP11A. Experiments were performed by Dr. Kent Rossman.

- **Other Achievements**

In addition to the research achievements described above, other accomplishments in the past 12 months include the publication of a review paper in the journal Small GTPases in March 2014. This review focused on the role of GAPs and GEFs in regulating RhoA- and Rac1-mediated cellular adhesion and migration – concepts that are intimately linked to the above research. The review reference is: Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases 2014; 5:e27958 (PMID 24607953). Support from the DoD was acknowledged.

I also attended two research conferences in the last year, which both afforded me invaluable opportunities to meet and discuss my research with relevant people in the field. The first was 'The Triangle Cytoskeleton Meeting,' an ASCB local meeting held at the Research Triangle Park, NC in September 2014. The second was the 2014 ASCB conference held in Philadelphia, PA in December. I presented a poster of my research at the latter conference. The poster abstract reference is: Lawson CD, Rossman KL, Fan C, Perou CM, Burridge K, Der CJ. The role of RhoGAPs in basal-like breast cancer. Mol. Biol. Cell 2014; 25:185-186 (Abstract P1855). Again, DoD support was acknowledged.

What opportunities for training and professional development has the project provided?

Under the co-mentorship of Drs. Der and Burridge, and with the help of their respective lab members and other researchers at UNC, this project has exposed me to new techniques, skills, and equipment and has therefore provided excellent training opportunities. Furthermore, I have actively engaged in other activities (e.g. seminars, lab meetings, individual study) that have enhanced my professional development.

The diverse research interests of the Der and Burridge labs allows me to interact daily with experts in particular topics and techniques and I have exploited the respective knowledge of each lab to receive one-on-one training that has greatly contributed to this project. For example, I have received training in performing in vitro growth assays (2D clonogenic, MTT, senescence) from experienced members of the Der lab, whereas members of the Burridge lab have tutored me in the performance of Rho GTPase pulldown assays as well as in fluorescent microscopy techniques. Training in the acquisition of fluorescent images has also been provided by a regional representative from Zeiss. In addition to members of the Der and Burridge labs, I have received training from other UNC-based researchers and facility staff. Notably, I have been taught how to perform time-lapse imaging by a graduate student in Dr. James Bear's lab using their Olympus Vivaview system. I have also received training from staff at UNC's Flow Cytometry Core Facility that has allowed me to perform cell cycle analyses.

The research environment at UNC provides not only excellent opportunities for training, but also a high-caliber platform for professional development in the form of regular lab meetings, journal clubs,

seminars, and visiting lecturers. At the lab level, the Der and Burridge labs both hold weekly lab meetings/journal clubs and these provide me with the chance to learn about my peers' research and to gain knowledge of their particular interests. Furthermore, I present my own research to both labs approximately every three months, which has provided constructive feedback as well as developing my presentation skills. I have also presented my research at a departmental level. Departmental seminars are held weekly and have allowed me to learn about other people's research at UNC. Seminars presented by visiting lecturers also occur frequently and afford useful insight into work being carried out at other institutions. Interacting with guest speakers, coupled to my attendance at two conferences (as described above) have provided me with the opportunity to form professional contacts on a local, national, and international level. Finally, through individual study over the past year, I have gained knowledge of new areas that are important to my research and which have complemented my pre-existing expertise. By reading peer-reviewed articles in the leading journals in cancer and GTPase biology, I have become familiar with topics such as proliferation pathways, the cell cycle, cytokinesis, and senescence.

How were the results disseminated to communities of interest?

The major mechanism through which these results have so far been communicated to relevant communities is by presentation of a poster at the 2014 annual ASCB conference. This opportunity allowed me to discuss my findings with experts in the field. These interactions were not only a chance to disseminate my results, but also stimulated conversation leading to valuable feedback that has influenced the direction of the project. To reach a far larger audience, I intend to submit the findings of this project for publication in a peer-reviewed journal within the next 6-12 months.

In addition to communication with other researchers within the field, I have also been involved with outreach activities to reach a more public audience. Notably, I have participated in 'open lab' events in which members of the public – largely cancer patients, survivors, affected families, and advocates – have been invited to tour the Der lab facilities and hear about the research that we do. Communication of our research to a lay audience in this manner is a critical way in which to increase public understanding, but also serves as an important reminder of the relevance of our research to the wider community.

What do you plan to do during the next reporting period to accomplish the goals?

The plan for the next 12 month research period is to:

- Perform in vitro Matrigel invasion assays on GAP-depleted cell lines.
- Perform in vivo tumorigenesis and metastasis analyses in immunocompromised mice.
- Define the contribution of Rho GTPase signaling to the growth and migratory phenotypes of GAP-depleted BLBC cell lines by performing RhoA/Rac1/Cdc42 pulldown assays, by attempting to phenocopy GAP-deficient responses with constitutively active Rho GTPase mutants, and by characterizing the ability of catalytically-inactive GAP mutants to rescue each phenotype.
- Repeat key experiments using the HCC1937 cell line.
- Examine ArhGAP11A and RacGAP1 protein expression in human breast tumor samples.
- Prepare manuscript and submit this research for publication.
- Attend and present data at conference(s), e.g. 'Regulation and Function of Small GTPases' FASEB Summer Research Conference in June 2015.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Once published, the results of this project are likely to impact upon the field of cancer biology in two key ways. Firstly, by reclassifying RhoGAPs as a class of molecule that can support oncogenesis, and secondly, by establishing ArhGAP11A and RacGAP1 as potential targets for pharmacological intervention in the treatment of BLBC.

Rho family small GTPases have been strongly linked to tumor growth and metastasis, typically through their aberrant activation by GEFs, which is generally thought to result in hyper-elevated Rho GTPase activity and therefore tumorigenesis. In contrast, RhoGAPs, which downregulate Rho GTPase activity and are relatively understudied in comparison to RhoGEFs, are generally presumed to act as tumor suppressors. The results of our study, which indicate that ArhGAP11A and RacGAP1 are overexpressed in BLBC and are essential for tumor growth, are contrary to the perceived notion that RhoGAPs can only act as tumor suppressors and indicate that certain GAPs can in fact promote tumorigenesis. These surprising findings should provoke a reassessment of the role of RhoGAPs in human cancer and may lead to additional studies with the potential to identify other RhoGAPs as drivers of cancer.

By identifying, validating, and characterizing ArhGAP11A and RacGAP1 as oncogenes in the development of BLBC tumor growth, our research has defined these proteins as novel molecular targets for the development of therapeutic strategies, with the potential to improve treatment of patients with this particularly aggressive subtype of breast cancer. Hence, our research will not only contribute to knowledge of our particular field but may also act as the basis for the development of improved, subtype- and molecularly- targeted cancer therapies.

What was the impact on other disciplines? Nothing to report.

What was the impact on technology transfer? Nothing to report.

What was the impact on society beyond science and technology? Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There have been no significant changes to the objectives or scope of the project. The problems and changes listed below are considered minor and have not altered the overall goals.

In the original SOW, it was stated that one of the breast cancer cell lines that would be used for experiments was the MDA-MB-231 cell line. However, it came to our attention that this cell line is now considered to belong to the 'claudin-low' subtype of breast cancer[9]. Hence, we chose to use SUM149 and HCC1937 cells for our studies as these are widely accepted to belong to the basal-like subtype.

It was originally intended to use soft agar assays to study cancer cell growth in vitro; however, we were unable to form colonies of parental SUM149 or HCC1937 cell lines under these conditions (despite being able to grow other, non-BLBC lines). We therefore decided to evaluate in vitro growth using 2D clonogenic and MTT proliferation assays on cell culture plastic instead. These conditions allowed for robust and reproducible growth of parental SUM149 and HCC1937 cells.

One of the sub-aims of major task 3 was to perform in vitro GAP activity assays to identify the GTPase-specificity of ArhGAP11A. However, other labs have subsequently identified that this GAP

predominantly acts on RhoA, but not Rac1 or Cdc42, in vitro[6-8]. Our own observations agree that RhoA is the major target, but also show a previously unidentified role for ArhGAP11A in inactivating Cdc42 (Fig. 8). We will verify which GTPases are targeted by ArhGAP11A in our BLBC cell lines. If we fail to identify a role for RhoA or Cdc42 in our experiments, then we will expand our in vitro GAP assay analysis to identify whether ArhGAP11A also has specificity for other, less well-studied GTPases. There are 20 human Rho GTPases, but the field is dominated by the study of RhoA, Rac1, and Cdc42. Hence, our in vitro GAP assay analyses have the potential to yield novel results.

Other changes in approach have included alterations to the relative timing of particular experiments. Notably, we decided to prioritize in vitro proliferation, cytokinesis, apoptosis, and migration assays (originally planned for year 2) over in vivo tumorigenesis and metastasis assays (planned for year 1). These experiments were reordered as it was logical to ascertain the mechanisms of the in vitro ArhGAP11A- and RacGAP1-related growth defects before doing mouse experiments. It is our intention to perform in vivo tumorigenesis and metastasis assays within the next 3-6 months.

Actual or anticipated problems or delays and actions or plans to resolve them
Problems, and the changes that have been applied to resolve them, have been addressed above.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
As noted above, the experiments with mice were not performed in the last 12 months as planned, but will be carried out within the next reporting period. There will be no changes to the approved protocols.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.
Lawson CD, Burridge K. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases 2014; 5:e27958.
(Published, federal support acknowledged)

Lawson CD, Rossman KL, Fan C, Perou CM, Burridge K, Der CJ. The role of RhoGAPs in basal-like breast cancer. Mol. Biol. Cell 2014; 25:185-86 (Abstract P1855).
(Published abstract, federal support acknowledged)

Books or other non-periodical, one-time publications. Nothing to report.
Other publications, conference papers, and presentations. Nothing to report.

- **Website(s) or other Internet site(s).** Nothing to report.
- **Technologies or techniques.** Nothing to report.
- **Inventions, patent applications, and/or licenses.** Nothing to report.
- **Other Products.** Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Campbell Lawson
Project Role:	PI
Nearest person month worked:	12
Contribution to Project:	All experiments and analysis
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Campbell Lawson (PI) – Nothing to report

Keith Burridge (Key personnel) – The grant '1RO1 GM29860-32' K. Burridge (PI) "Cell Adhesion and the Regulation of Rho GTPases" 04/01/1981-08/31/2014 from NIH/NIGMS was awarded a one year no cost extension. A renewal of this grant was submitted in July 2014 and scored an 11%. It is anticipated that this will be renewed and will go from 04/01/15- 03/31/19. The goals of this grant are to understand how adhesion molecules regulate the activities of Rho GTPases, and how mechanical forces applied to cell adhesion molecules affect Rho GTPase activity and function. All other funding for Keith Burridge has remained the same as when this fellowship was submitted.

Channing Der (Key personnel) – The following grant has been awarded:

Name of Principal Investigator	Title of Project	Funding Agency	Grant Term	Amount of Funding	Percent Effort of Contact PI	List of Specific Aims as Stated in Grant Proposal
Der, Channing	Identification and validation of Raf inhibitor-based combination KRAS-targeted therapies	Lustgarten Foundation	7/1/2015-6/30/2018	$974,413 (Total direct)	25%	To: (1) identify combination inhibitor strategies to render Raf inhibitor therapy cytotoxic; (2) identify drivers of resistance to Raf inhibitor therapy; and (3) determine the selectivity and anti-tumor activity of Raf inhibitor-based combination therapies

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

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